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Usefulness of oils for cleaning the host matrix and for cryoprotection of lipidic cubic phase crystals

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The lipidic cubic phase method is an effective approach for membrane protein crystallography. The *in meso* grown crystals are usually cryocooled directly without removing the host matrix from the harvested crystal surface. However, the host matrix often causes the appearance of scattering rings and an increase in background scattering during the data collection. Moreover, the frozen host matrix sometimes becomes opaque and it can hinder conventional crystal centering. In this study, several oils were examined for their ability to clean the host matrix and to provide cryoprotection for crystals grown in the lipidic cubic phase. Several of the tested oils appeared to be useful in terms of their effect on crystal stability and background scattering. This method should be of value for the collection of highly accurate data sets.

1. Introduction

Membrane proteins play essential roles in various biological processes and have therefore attracted interest as potential drug targets. The lipidic cubic phase (LCP) or *in meso* method is an approach for crystallizing membrane proteins (Landau & Rosenbusch, 1996; Pebay-Peyroula *et al.*, 1997; Cherezov *et al.*, 2007; Caffrey, 2015). This method provides type I crystals that are typically small but well diffracting (Michel, 1983). The crystals are grown in a host matrix, which is a material composed of water and lipids such as monoolein.

The *in meso* crystals are usually cryocooled directly after the harvest, because the host matrix acts as a cryoprotectant. Under some conditions, however, scattering rings are observed from the host matrix and ice. Moreover, the frozen host matrix sometimes becomes opaque in nylon loops. Sometimes the crystals obtained by the LCP method are very small and thus can be difficult to center using optical microscopy during a diffraction experiment. To overcome this situation, various methods have been proposed. The most popular method is using a micro-focused X-ray beam, such as the rastering for crystal mapping and the X-ray mesh scanning methods (Hilgart *et al.*, 2011; Melnikov *et al.*, 2018; Hirata *et al.*, 2019). However, the availability of beamlines at which these methods using a micro-focused beam can be carried out is limited, and optical microscopy is still used during the crystal centering step at many beamlines. The crystal centering problem can thus impede the experiment and can also contribute to decreased diffraction intensity and increased background scattering. In some cases, the host matrix is removed from the crystals prior to data collection (Nollert & Landau, 1998; Luecke *et al.*, 1999).

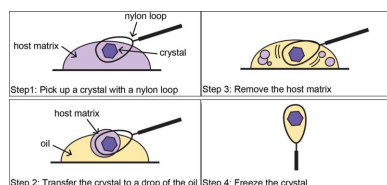


Table 1
Summary of oils used in this study.

Compound name	Supplier	Density (g ml ⁻¹)	Viscosity (cP)
Squalane	Sigma (St Louis, MO, USA)	0.81	27
Squalene	Sigma (St Louis, MO, USA)	0.86	12
Phytantriol	Avanti Polar Lipids (Alabaster, AL, USA)	0.93	—†
Paratone-N	Hampton Research (Aliso Viejo, CA, USA)	0.87	550
Liquid paraffin	Nacalai Tesque (Kyoto, Japan)	0.87	30
Low-viscosity liquid paraffin	Nacalai Tesque (Kyoto, Japan)	0.82	3.4

† Though the viscosity of phytantriol is unknown, it has high viscosity comparable to that of Paratone-N.

In data collection from soluble protein crystals, immiscible oils are sometimes used for cryoprotection (Riboldi-Tunnicliffe & Hilgenfeld, 1999). These oils are suitable for crystals obtained under a wide range of crystallization conditions. In addition, the application of oils has been proposed for removing the host matrix from crystals obtained by the LCP method (Caffrey & Cherezov, 2009). However, there has been no report of a systematic study of the use of oils for removal of the host matrix for cryoprotection, and the usefulness of oils in these contexts is unknown. In this study, we examined the effectiveness of several oils in the cryoprotection of crystals obtained by the LCP method in terms of the crystal quality and the facilitation of the experiment.

2. Materials and methods

2.1. Materials

The following six oils were chosen for these experiments: squalane, squalene, phytantriol, Paratone-N, liquid paraffin and low-viscosity liquid paraffin. The density and viscosity of each of the oils as given in their respective catalogs are listed in Table 1. Loops made with 20 µm-diameter nylon fibers were purchased from Hampton Research (Aliso Viejo, CA, USA).

2.2. Background scattering

For each of the oils, a small amount of oil was mounted on a nylon loop. The oil was then flash-cooled in a flowing nitrogen stream at a set point of 100 K. X-ray diffraction images of each oil were collected at 100 K.

2.3. Crystal stability

The effects on crystal stability were estimated using bacteriorhodopsin (bR) crystals which belong to space group *P*₆. This type of bR crystal has typical type I packing in which 2D layers of membrane proteins stack into a 3D crystal. bR was purified and crystallized as described previously (Hasegawa *et al.*, 2018). Briefly, the purple membrane was purified from *Halobacterium salinarum* strain R1 (JMC9409) by a standard procedure (Oesterhelt & Stoebenius, 1971). bR was solubilized at 1.2% (w/v) β-octyl glucoside (Anatrace, Maumee, OH, USA) from the purple membrane. Crystals

were obtained by the LCP method using monoolein as host matrix containing 1.0% (w/w) squalane (Sigma-Aldrich, St Louis, MO, USA) and 5% (w/w) trehalose C16 (Dojindo, Kumamoto, Japan) as additives. Crystals were grown for more than three months in a precipitant solution containing 2–2.5 M Na/K phosphate (pH 5.6). Crystals with a largest dimension of 50–100 µm and a thickness of 10–20 µm were selected for the experiments, because this is a typical size for crystals obtained by the LCP method. The crystals and surrounding lipids were picked up with a nylon loop and transferred to a 1 µl drop of the respective oil. Crystal cleaning was performed with the nylon loop by moving the crystal gently back and forth in the oil drop. The crystals were kept within the oil drops for 60 min. Photographs of the crystals were taken at 1, 5 and 60 min after transferring the crystal to the drop of oil.

2.4. Data collection

Initial diffraction experiments with all oils were performed using an in-house X-ray source with bR crystals cooled at 100 K. Crystals of ~80 µm were transferred and cleaned in each oil. The crystals in the oil were immediately flash-cooled in a nitrogen gas stream at 100 K. The steps of this method are shown in Fig. 1. Two diffraction images at two different angles 90° apart were collected with an oscillation angle of 1° and an exposure time of 5 min. Further diffraction experiments with the selected oils were performed at BL41XU of SPring-8 (Hyogo, Japan). Crystals of 50–150 µm with the host matrix

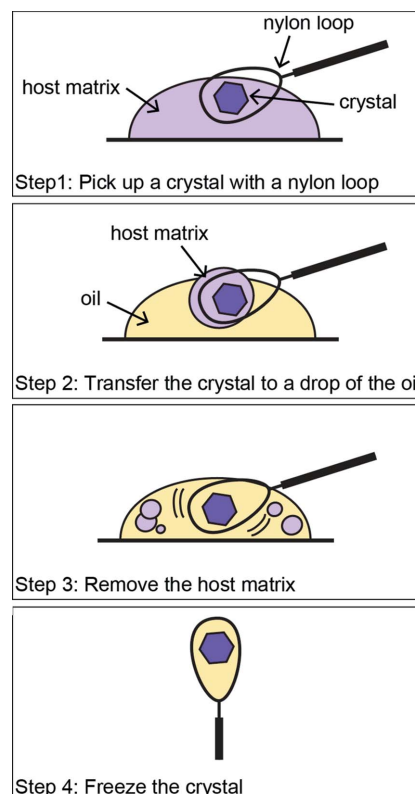


Figure 1
Steps of the crystal cleaning method.

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and treated with the respective oils were flash-cooled in a nitrogen gas stream at 100 K. Data collections were performed with an oscillation angle of 0.2° and an exposure time of 0.2 s for one frame, and a total of 500 frames were measured from each crystal with the selected oils. The wavelength of X-rays and the beam size were set to 0.80 \AA and $35 \times 22 \text{ }\mu\text{m}$, respectively. The photon flux was set to $2 \times 10^{10} \text{ photons s}^{-1}$ by using aluminium attenuators. Diffraction spots were recorded with an Eiger X 16M detector (Dectris). The distance between the sample and detector was set to 135 mm. Crystals were cooled at 15 K with a helium gas stream during the data collection. The data sets were processed using the *XDS* program (Kabsch, 2010). The appropriateness of data processing was evaluated by the value of $(I/\sigma)^{\text{asymptotic}}$, which is given as *ISa* in the *XDS* program (Diederichs, 2010). Data sets at $(I/\sigma)^{\text{asymptotic}} < 10$ were excluded from further analysis. The resolution limits were defined from $\text{CC}_{1/2} \simeq 50\%$. The Wilson *B* factor, which reflects the diffraction quality of the crystals, was calculated using the *truncate* program (Dauter, 2006) in the *CCP4* program suite (Collaborative Computational Project, Number 4, 1994).

3. Results

3.1. Background scattering from oils

Diffraction data were collected from a small amount of each oil as well as from the host matrix across the nylon loop. In the case of phytantriol and Paratone-N, it is difficult to minimize the amount of liquid in the loop because of their high viscosity. A broad diffuse scattering ring was found in the range of $5\text{--}4.2 \text{ \AA}$ in the diffraction pattern of each oil (Supplementary Fig. S1). In the diffraction image of phytantriol, this broad ring was especially strong, and an additional broad ring was observed in the low-resolution region of $\sim 20 \text{ \AA}$. In the diffraction image of low-viscosity liquid paraffin, additional sharp rings were observed at around 4.1 and 3.6 \AA . From these facts, we concluded that background scattering was negligible in squalene, squalene, Paratone-N and liquid paraffin but non-negligible in phytantriol and low-viscosity liquid paraffin.

3.2. Effect on crystal stability

All oils were able to remove the host matrix from the crystal surface. The required time for cleaning was 1–5 min. Low-viscosity liquid paraffin dissolved and removed the host matrix most rapidly. Owing to their high viscosity, phytantriol and Paratone-N stripped the host matrix away from the crystals rather than dissolving it. Cleaning was most easily achieved with phytantriol and Paratone-N, and the second best were squalene and low-viscosity liquid paraffin.

After cleaning, these crystals were kept in their respective oil drops (Supplementary Fig. S2). At 5 min, the edges of the crystals in squalene and low-viscosity liquid paraffin were dissolved. In the drop of phytantriol, the oil surrounding the crystals became clouded [Supplementary Fig. S2(c)]. No significant changes were observed in the other oils. After 60 min, the crystals survived and retained their shape with the

phytantriol and Paratone-N. However, the crystals cracked in the other oils. Squalene and low-viscosity liquid paraffin especially affected the crystals: the crystals disintegrated completely in these oils [Supplementary Figs. S2(b) and S2(f)]. In the drop of low-viscosity liquid paraffin, the water droplets merged to large droplets.

3.3. Diffraction experiment

The time required for cleaning and freezing one crystal was ~ 3 min. When phytantriol and Paratone-N were used, it proved very difficult to pick up the crystal and a small amount of oil in the loop owing to their high viscosity. Diffraction spots were observed for all crystals with oils as well as for the crystal in the host matrix which was directly picked up from the LCP (Supplementary Fig. S3). Ice rings were not found in any of the diffraction images. For squalene, phytantriol, Paratone-N and liquid paraffin, the resolution and mosaicity were comparable to those in the untreated crystal. On the other hand, the resolution was decreased with squalene and low-viscosity liquid paraffin. Moreover, the mosaicity was severely increased with low-viscosity liquid paraffin [Supplementary Fig. S3(g)]. From these results, we concluded that crystals in squalene and low-viscosity liquid paraffin deteriorated quickly.

3.4. Data collection at the synchrotron facility

On the basis of these results, we selected squalene, Paratone-N and liquid paraffin for further assessment. As mentioned in Section 3.3, it was very difficult to mount crystals properly with Paratone-N. Moreover, the treatment with Paratone-N became more difficult when the crystals were smaller. In the case of squalene and liquid paraffin, even small crystals were picked up easily from the oil drops, and it was easy to align the orientation of the plane of the crystals to the nylon loops. The crystals treated with the respective oils in the nylon loops are shown in Fig. 2. In the case of no treatment, the crystals were difficult to observe with a microscope from the perpendicular direction of the nylon loop plane owing to the thick and opaque host matrix. For squalene, the crystal position was clearly visualized from an in-plane direction and the perpendicular direction of the nylon loop plane, which

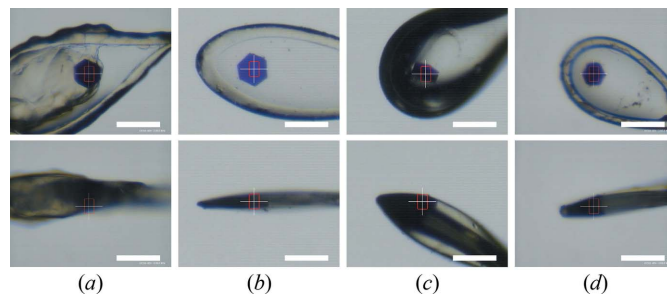


Figure 2
The crystals used for data collection with (a) the host matrix, (b) squalene, (c) Paratone-N and (d) liquid paraffin. The scale bars indicate 0.1 mm .

helped in the crystal centering. For Paratone-N, the crystal position was clearly visualized from an in-plane direction of the nylon loop plane. However, the crystal position was not clear from the perpendicular direction of the nylon loop plane owing to the large volume of Paratone-N [Fig. 2(c)]. Thus, the

crystal centering was difficult. For liquid paraffin, the crystals were clearly observed, as when using squalane.

Full data sets were collected from these crystals. All crystals diffracted to high resolutions of 1.5–2.2 Å (Fig. 3 and Supplementary Tables S1–S4). The mosaicity is related to the crystal quality and often increased during cryoprotectant treatment (Pflugrath, 2015). The average mosaicities for the crystals treated with oil were 0.13–0.15°. These values are comparable to that of $0.13 \pm 0.05^\circ$ for the untreated crystals [Fig. 4(a)]. The average Wilson B factors, which are also related to the crystal quality, were 26–27 Å² for the crystals treated with oil and also comparable to that for the untreated crystals of 23 ± 3 Å² [Fig. 4(b)]. From these facts, we concluded that the crystals retained their quality during the oil treatment.

4. Discussion

The method assessed herein was actually used in our recent studies for bR and heme A synthase (HAS) (Hasegawa *et al.*, 2018; Niwa *et al.*, 2018). In the case of bR, squalane was used for cleaning and cryoprotection (Hasegawa *et al.*, 2018). The size of the crystals used by Hasegawa *et al.* was 300–400 µm, which was larger than the size of the crystals used in this work. However, the experiment was successful. In previous studies on bR, the host matrix has been removed from the crystal surface by means of enzymatic digestion (Nollert & Landau, 1998) or detergent solubilization (Luecke *et al.*, 1999). Compared with these previous approaches, our method can significantly reduce the treatment time from several hours or overnight to 3 min (Table 2). Moreover, our structures had the lowest average B factor, indicating that the crystal quality was maintained during the oil treatment.

In the case of HAS, the structure of HAS was unknown and thus data sets for the experimental phasing were required. Moreover, the HAS crystals were small (~20 µm) and twinned. Therefore, we collected the full data set of HAS from one crystal using this oil cleaning method in order to facilitate

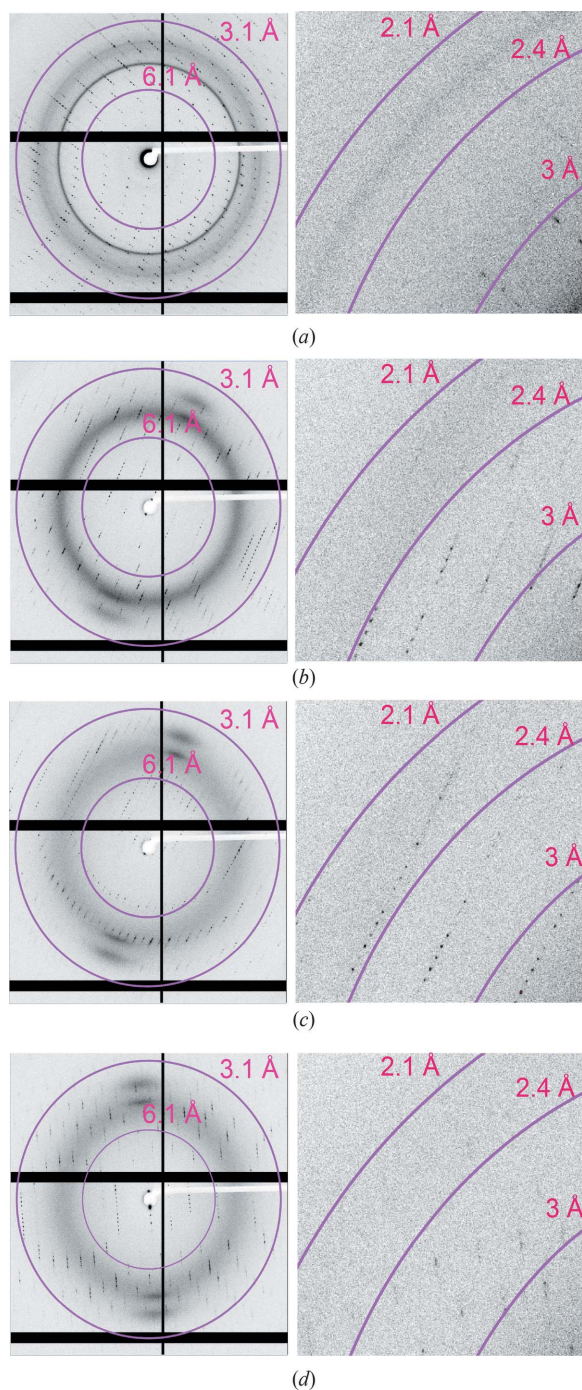


Figure 3
The diffraction images of the crystals with (a) the host matrix, (b) squalane, (c) Paratone-N and (d) liquid paraffin. The images were prepared by merging five consecutive diffraction images (corresponding to 1.0° rotation). Views of the low-resolution region are shown in the left panels and magnified views of the high-resolution region are shown in the right panels.

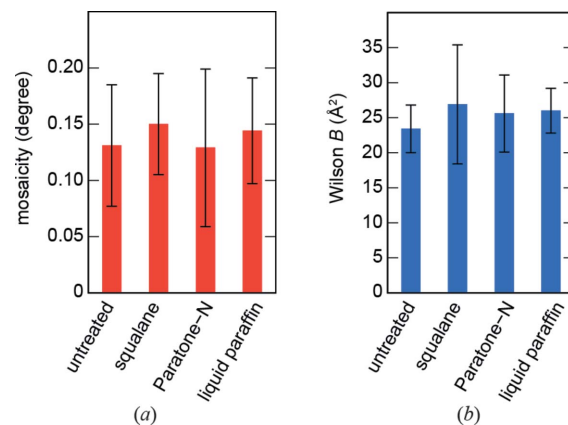


Figure 4
Effect on the crystal and data quality. (a) The mosaicities and (b) the Wilson B factors for the crystals treated with each of the oils are shown. Error bars indicate standard deviations for the values shown in Supplementary Tables S1–S4.

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Table 2

The required time and the average *B* factor of each method.

Method	Reference	Required time	Average <i>B</i> factor (\AA^2)†
Enzymatic digestion	Nollert & Landau (1998)	Overnight	35.0 (PDB ID 1qhj)
Detergent solution	Luecke <i>et al.</i> (1999)	Several hours–overnight	30.0 (PDB ID 1c3w)
Oil cleaning	This work	~3 min	23.0 (PDB ID 5zil)

† The values are from the PDB entry in parentheses (Belrhali *et al.*, 1999; Luecke *et al.*, 1999; Hasegawa *et al.*, 2018).

crystal centering and to decrease the background scattering. Squalane was also used for the harvest and cryoprotection because Paratone-N was not suitable for such small crystals. We found that the small crystals of HAS were stable in squalane for a short period of time. The crystal centering was possible since the oil on the nylon loops was completely transparent and the thickness of the oil film was appropriate. In the data collection from small crystals, the multicrystal data collection strategy is usually used to increase $I/\sigma(I)$ and the completeness of the data set (Zander *et al.*, 2015). However, the merging of data from many crystals often affects the data quality indicators, such as R_{merge} . In addition, when crystals are twinned or have low isomorphism, the merging method is difficult. In such situations, our method may be useful to collect full data sets from one crystal.

Most of the target proteins have no color, and the crystal centering problem is more severe for colorless crystals. The application to lysozyme crystals obtained by the LCP method (Aherne *et al.*, 2012) as a model of colorless membrane protein crystals is shown in Supplementary Fig. S4. The oil-treated crystals were clearly visible, while untreated crystals were difficult to observe [Supplementary Fig. S4(a) and S4(b)]. Moreover, the scattering rings in the diffraction images were suppressed by oil treatment [Supplementary Figs. S4(c) and S4(d)]. Therefore, we conclude that this method is also useful for colorless crystals.

Squalane, Paratone-N and liquid paraffin did not affect crystals during harvesting, cleaning and flash-cooling. Paratone-N removed the host matrix most easily, while squalane and liquid paraffin were the easiest to use to mount and center crystals. On the basis of these results, it is advisable to test the oils and select the appropriate one for the experiment. This method will benefit studies of these crystals because correct centering contributes not only to the improvement of the signal-to-noise ratio but also to increasing the completeness of the data. Although membrane protein crystallography is challenging, we believe that this method will be of help in overcoming some of the difficulties.

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